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(72) Inventor RALPH BUCHANAN LLOYD GWATKIN

(19)

(54) THE IMMUNOLOGICAL CONTROL OF FERTILITY

(71) We, MERCK & CO INC., a Corporation duly organized and existing under the laws of the State of New Jersey, of Rahway, New Jersey, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention is concerned with the immunological control of fertility utilizing the antigenic, non-cellular proteinaceous coating of the mammalian egg, viz, the zona pellucida.

The possibility of utilizing immunological procedures for fertility control both in the male and in the female has been much discussed, but to date none has proved feasible. Such prior attempts are characterized by an inability to isolate an antigenic material in pure form, which is a necessary condition for the production of tissue-specific antibodies. Another shortcoming of such prior attempts is that antisera substantially specific to a certain genital tissue are species-specific, that is, such antisera give no evidence of immune reaction with the analogous tissue from distinct animal species; such results additionally characterize the species-specificity of prior antigen preparations. The requirements imposed upon any immunological approach to fertility must include tissue-specificity and cross-reaction (as opposed to species-specificity) because unless there is tissue specificity the immunological procedure could not be considered safe and without cross-reaction any such immunological procedure would be extremely limited since the antigenic source material would have to be obtained from the species to be rendered infertile — an impossibility for fertility control of the human female.

The possibility of immunological control of fertility using the antigenic mammalian zone pellucida has been considered. This structure, the zona pellucida, and the phenomena associated with it, including the "zona reaction", have been much studied in reproductive biology with respect to certain sperm-egg interactions: *approach, attachment* and *penetration. Approach* of sperm to egg appears to be a random process without numerical limit. *Attachment* (sperm to zona pellucida) appears to be attributable to definite sperm receptor sites on the surface of the zona and in all likelihood is limited numerically by available area. *Penetration,* however, is normally limited to a single sperm. On penetration the receptor sites are deactivated (zona reaction). If the receptor sites or sites peripheral thereto are antigenic, the possibility of creating antibodies which mask or alter the zona pellucida and thus preclude attachment and penetration is an attractive, non-hormonal approach to fertility control. But to date such an approach has fallen to the above discussed two hurdles, namely; species-specificity and lack of tissue-specificity. While Applicant is bound by no theory, it would appear that such prior attempts were unsuccessful principally because the precise antigenic material critical for practice of the present invention was not obtained in functional form by prior procedures, which typically comprise gross saline extraction of macerated whole ovarian tissue. Antisera prepared from such saline extracts, although rendered to some degree ovary-specific by adsorption of unwanted antibodies by contacting with diverse somatic tissues, are invariably found to be species-specific; further it is extremely doubtful that such prior antisera are even capable of entering into an immune response with the critical antigenic sites of the zona pellucida for reasons which will be made evident below.

The present invention is based on the unexpected discovery that a tissue specific, non-species dependent (cross-reacting) anti-fertility vaccine can be prepared from the mammalian zone pellucida by heating either zonae pellucidae *per se,* intact eggs or ovarian tissue comprising eggs in an appropriate medium such as water such

that the proteinaceous zona pellucida is solubilized. Such aqueous solutions comprising solubilized zona pellucida may be evaporated to dryness to provide a form of the zona protein which is readily again solubilized in aqueous solution. Thus in the present specification the term "solubilized zona pellucida" (SZP) means mammalian zona pellucida protein — dry or in solution — that has been heated at a temperature in the range 65 to 100°C and thereby rendered water-soluble. The present invention provides SZP. Antibodies prepared from such SZP are not species-specific and specifically react with the zona pellucida of the mammalian animal being treated for fertility control.

The invention further provides a vaccine for immunological control of fertility in the female mammal comprising solubilized zona pellucida as hereinbefore defined together with a carrier or diluent, an antiserum composition comprising the antibody anti-solubilized zona pellucida together with a carrier or diluent; and an injectable pharmaceutical composition comprising anti-solubilized zona pellucida for control of fertility in the female mammal together with an injectable carrier or diluent.

The present invention also provides a process for preparing an anti-fertility vaccine comprising solubilizing zona pellucida in an aqueous medium by heating the zona pellucida at from 65 to 100°C. and dissolving the resulting product in water.

The present invention also provides a method for the immunological control of fertility in the non-human female mammal and comprising administering by injection solubilized zona pellucida as hereinbefore defined and a method for the immunological control of fertility in a non-human female mammal comprising administering by injection anti-solubilized zona pellucida.

The following specific characterizing data and techniques representatively state the general principles involved in practising the first-described aspects of the present invention and from such representative teachings one will be enabled to practise the present invention.

The vaccine of the present invention provides immunological control of fertility when administered to the female mammal by methods hereinafter described. The vaccine comprises solubilized mammalian zona pellucida derived from a non-self source. The non-self source requirement exists because eggs, unlike sperm, are present early in foetal development and thus the female is tolerant to its own zonae pellucidae, the non-cellular proteinaceous coating which surrounds the mammalian ovum. As described below, there is no criticality as to the source of the zona pellucida for practice of the present invention with the sole proviso given above that the solubilized zona pellucida be obtained from an animal species distinct from the one to be treated for fertility control. Thus for example, for immunization of the human female, practical source animals for zonae pellucidae include practically any lower mammalian species such as the laboratory hamster or laboratory mouse, but for practical reasons slaughterhouse sources such as cow, sheep and pig may be preferred depending on availability. The vaccine comprises an aqueus solution of the solubilized mammalian zone pellucida (SZP), either alone or admixed with suitable adjuvant such as aluminium hydroxide, mineral oil-water emulsion or emulsifiers such as calcium alginate. The particular adjuvant is not critical. The vaccine composition may be prepared in unit dosage form or in bulk form for ultimate dilution. Alternately, the vaccine may be prepared in dry form (lyophilized) from the solubilized form for convenience of storage.

The dosage form of the vaccine may be prepared from the bulk solution by dilution with sterile water or with buffered aqueous adjuvant (preferred pH 7.0 to 7.2) such that the unit dosage by injection comprises SZP in the range of from 1.0 to 10.0 μg. per kg. of body weight. Method of treatment aspects for effective vaccination, i.e., period and number of unit injections, will be described below.

The antisera compositions of the present invention comprise antibodies against the antigenic zona pellucida and such compositions are useful as a passive means of immunological control of fertility since inoculation with such antibodies provides short term infertility and additionally appears to prevent uterine implantation of fertilized ova. The methods of preparing such antisera and methods of administering it are described below. Compositionally the antisera comprise the identified antibody in cell-free sera or fractions thereof (Cohn fractions or lyophilized form).

Well-known analytical techniques for characterizing the quality and quantity of such vaccine and antibody preparations, anti-SZP, include for example: 1.) Gel Immunodiffusion [Ouchterlony, *26, Acta, Pathol, Microbiol, Scand,* 507 (1949)]; 2.) Sandwich Technique, using Fluorescent Antibody [Coombs, *Immunological Methods;* ed. by Ackroyd; Blackwell; Oxford (1964)]; and 3.) Inhibition of In Vitro Fertilization [Gwatkin, et a., *30 J. Reprod. Fert.* 389 (1972)].

In the method of treatment using the composition of the present invention it is to be emphasized that the precise dosage level and period of inoculation depend upon the case history of the individual being treated and in the last analysis are left to the routine determination of the skilled therapist because such parameters are readily determined by the general guidelines given here. In general, vaccination with the vaccine comprising solubilized mammalian zona pellucida provides for a reversible state of infertility of the female being treated typically lasting from 15 to 30 weeks. Typically, immunization (infertility) in the human female is achieved by injection with vaccine solution comprising from 70 to 700 μg. of SZP given once or repeated after an interval of from 7 to 14 days. The state of immunity and therefore of infertility may be determined by obtaining a small blood sample and titrating the serum portion thereof by gel immunodiffusion against SZP. Agglutination at a titer of greater than 1:16 indicates a sufficiently high antibody level to insure infertility. The return of fertility is indicated by a sharp drop in antibody titer.

Treatment by administering the above-described antisera is, in the case of treatment of the human female, by injection of a globulin-rich fraction of serum from an immunized animal such as the horse, sheep or pig. Such a fraction is prepared by a cold-alcohol precipitation (Cohn method) and typically the unit dose by injection comprises from 10 to 20 mg./kg. body weight. One injection given up to say 14 days after coitus with the possibility of conception is typically sufficient to prevent pregnancy, although repeated injections given daily up to the fourteenth day give added assurance of effectiveness. Alternately the anti-SZP compositions may be used as a means of short-term control of fertility. Unit dosage by injection of from 10 to 20 mg./kg. body weight of the above-described fraction provides an infertile state lasting at least 5 days, the return of fertility being determined as described above.

As previously indicated there is no criticality as to the source of the mammalian zona pellucida, but for practical reasons the following species of animals are preferred for the harvest of zonae pellucidae for the preparation of vaccine to be used in humans and in domesticated animals: laboratory animals such as mice, hamsters, rats and rabbits and slaughterhouse animals such as cow calves, sheep and pigs.

Solubilization of the zona pellucida is achieved by heating intact zonae at from 65° to 100°C in an aqueous medium comprising phosphate buffered saline (PBS, having the composition in g/l: $CaCl_2$ 0.1; KCl 0.2; KH_2PO_4 0.2; $MgCl_2 \cdot 6H_2O$ 0.1; NaCl 8.0; $Na_2HPO_4 \cdot 2H_2O$ 1.15) or similarly buffered saline solutions providing a pH of from 7.0 to 7.4. As a practical matter the solubilization of the proteinaceous zona pellucida may be performed directly upon gross ovarian tissue that has been mechanically comminuted prior to heating. Preferably, the ovarian tissue is obtained from immature animals since the number of zonae-bearing oocytes decreases rapidly with age. Alternatively, and in the case of small scale preparations, the zonae may be mechanically separated from the ova. This may be accomplished, for example, by drawing the complete ovum through an aperture approximately 1/2 to 3/4 its normal diameter. By such a process the zona is ripped from the ovum as it is pulled through the aperture.

The zona pellucida thus solubilized may be further isolated or purified from contaminating matter by centrifugation and dialysis of the resulting clear solution through Cellophane in an aqueous bath, followed by column chromatography ("Cellophane" is a Trade Mark). Active SZP fractions off such a column are identified by standard procedures previously enumerated such as immunodiffusion and *in vitro* fertilization assay.

The purity of the resulting SZP may be determined by any of the following well-known procedures:
1.) Ultracentrifugation; 2.) Electrophoresis on Agarose-Acrylamide Composite Gels [7 *Biochemistry* 668 (1968)]; 3.) Inhibition of Hamster Fertilization *in vitro*; and 4.) Immunodiffusion against antiserum specific for the zona protein.

In general the antisera compositions of the present invention are prepared by injecting into a host animal the solubilized zona pellucida of a distinct donor species and thereafter collecting serum antibodies. More particularly the following procedure is carried out: A suitable animal such as a horse, sheep or goat receives an intramuscular injection of SZP in solution (5—10 ug. SZP/Kg. body weight) in 0.5—1.0 ml. complete Freund's adjuvant). Starting 10 days later similar injections are given in incomplete Freund's adjuvant at 10 day intervals. The animals are bled at these times and the titer of antibody is measured by immunodiffusion against purified SZP.

The following examples specifically illustrate but do not limit the present invention. In fact the following specific

examples describe procedures which are generally applicable requiring only non-critical modifications to practise the invention.

EXAMPLE 1

Preparation and Properties of Solubilized Hamster Zona Pellucida and Analytical Techniques

Hamster zonae are isolated from intact ova by a rupture technique. In this technique individual ova are drawn through a narrow aperture (60 μ bore pipette) such that the zona coat ruptures and separates from the intact ovum (vitellus). Two thousand zonae pellucidae thus obtained are placed in 100 microliters of aqueous phosphate buffered saline [PBS; *99 J. Exp. Med.* 167 (1954)] and heated at 65°C. for 35 minutes whereupon the zonae are completely dissolved to provide a clear solution. Ultracentrifugal analysis shows the solution to contain a single molecular species with a molecular weight of 8.9×10^6. The molecular species is a protein, consisting of 17 amino acids. (See the Table).

TABLE

Amino Acid Composition of Hamster Zona Protein

Amino Acid	% Total Amino Acids
TYR	9.31
PHE	10.40
LYS	6.78
HIS	4.88
ARG	2.11
ASP	4.27
GLU	7.77
THR	6.99
SER	6.71
PRO	5.89
ALA	5.23
GLY	6.54
VAL	7.25
CYS	0.92
MET	0.32
ILE	0.24
LEU	14.38

Consistent with the amino acid composition, the hamster zona protein (hamster-SZP) is found to be neutral or weakly basic. Gel electrophoresis of the hamster-SZP protein thus obtained (10% polyacrylamide; pH 7.0; 2 milliamps per 5 mm diameter tube) fails to move the protein after 18 hours — the sample remaining as a single band at the top of the gel column. The band stains deeply with protein stain (fast green). The band does not stain with alcian blue, suggesting that the SZP protein contains little or no carbohydrate. On treating the hamster-SZP with urea and mercaptoethanol, it dissociates into three lower sub-units of molecular weights 100,000, 80,000 and 55,000 as revealed by migration in the above-described electrophoresis gel; the disassociation is effected by heating at 50°C. for 45 minutes a mixture of 10 μl of hamster-SZP, one drop glycerol and 10 μl of a solution, which comprises 100 mg. sodium dodecyl sulfate, 2.4 g. urea, 100 μl 1.2% by weight aqueous mercaptoethanol, and 10 ml. distilled water; thereafter the mixture is applied to a 10% by weight gel for electrophoresis against reference standards.

An analytical procedure for determining the activity of the hamster-SZP is by assessing its interaction with capacitated hamster spermatozoa. By capacitation is meant sperm which are capable of penetrating the zona pellucida for ultimate fertilization of the egg. Capacitation of sperm *in vitro* is routinely effected by treating sperm obtained from the epididymis for 6 hours in a tissue culture medium containing cumulus cells obtained from the oviduct of super ovulated animals 20 hours after the injection of 30 I.U. HCG [Gwatkin, et al., *30 J. Reprod. Fert.* 389 (1972)].

When such capacitated spermatozoa are incubated with hamster-SZP, the extent to which the spermatozoa are incapable of penetrating cumulus-free eggs is a measure of the activity of the SZP. For example, the activity of the abovedescribed hamster-SZP is determined by adding 10 μl of the solution comprising it to 30 μl of the solution comprising the capacitated hamster spermatozoa, 5×10^6 sperm per ml. After a 30-minute incubation, 20 cumulus-free hamster eggs are added and sperm penetration is scored after a 90-minute interval. Capacitated spermatozoa pretreated with hamster-SZP, although motile, are completely incapable of penetrating the eggs. When mouse-SZP obtained from the laboratory mouse by an identical procedure is substituted for the hamster-SZP, positive results are obtained in the above-described assay. This provides evidence that SZP is not species-specific. A second, direct, indication of cross-reaction (lack of species specificity) is provided by immunization against fertility, described below, wherein it is shown that vaccination of distinct animal species, ranging from the laboratory mouse to various primates, with hamster-SZP renders such animal species infertile as observed on natural matings and as observed by immuno-fluorescence of eggs taken from laparotomized species which had been vaccinated (primates).

Another analytical assay comprising

immunofluorescence of eggs treated with antisera against solubilized zona pellucida also indicates the lack of species specificity of the vaccine. Thus, for example, antiserum prepared in mice against the above-described hamster-SZP reacts with eggs of hamster, mouse, cow, rhesus monkey and squirrel monkey. Said reaction being evident when the eggs are treated with fluorescent anit-mouse globulin; however the concentration of the antiserum required to produce fluorescence varies with the species.

Vaccination of female hamsters with the above-described hamster-SZP produced, as expected, no detectable immune response, but when female mice are given vaccine injections (the vaccine comprising 4 μg hamster-SZP in 0.5 ml. Freund's Adjuvant — complete first injection, incomplete for subsequent injections) intramuscularly and again at 10-day intervals for a total of 4 injections serum analysis, as described above, shows that antibodies against the zona pellucida are established. When these mice are mated, the animals are found infertile.

To demonstrate the lack of species-specificity in the immune response, antisera collected from the vaccinated mice are collected according to the following procedure: a glass micro-pipette is inserted into the orbital sinus and blood is allowed to flow into the pipette. This blood is transferred to a glass tube and allowed to clot and then centrifuged at 1,000 g. for 5 minutes. The serum is decanted and stored at 5°C.

When such sera comprising anti-SZP antibodies are assayed for interaction with species-diverse mammalian eggs (hamster, mouse, cow, squirrel monkey, and rhesus monkey), a bright fluorescent is observed on the zona pellucida of eggs so treated when exposed to fluorescent anti-mouse globulin. However, the antibody-antigen reaction is specific to the zona pellucida structure for when the same antiserum is contacted with diverse tissues such as hamster cornea, hamster eggs without zona, hamster cumulus cells and hamster sperm no immune reaction is seen. These results constitute yet another analytical means for assaying vaccine and antisera preparations and in determining the effectiveness of vaccination. A further analytical procedure for determination of purity of the solubilized mammalian zona pellucida is to conduct immunodiffusion and immunoelectrophoresis evaluations in which the interaction of the SZP with an antibody produced from it is observed. With respect to the above-described hamster-SZP and the antiserum, it is observed that a single precipitin band is formed, thus indicating that the solubilized zona pellucida consists of a single antigen.

EXAMPLE 2

Isolation of SZP from Ovaries and Vaccine Preparation

Whole ovaries (100) from cow calves (ranging in age from 5—7 months) are collected on ice from the slaughterhouse. Each ovary comprises approximately 180,000 follicles from which approximately 2.0 mg. of zona protein is present. The ovaries are minced with scissors and for each gram wet weight thereof is added 5.5 ml. of Dulbecco's Phosphate Buffered Saline (PBS) [*99 J. Exp. Med.* 167 (1974)]. The mixture is homogenized for 1 minute in a Sorval Omnimixer cooled with ice. The homogenate is poured off into 25 ml. silica glass centrifuge tubes, which are placed in a boiling water bath for 15 minutes. The tubes are then centrifuged at 12,000 g. for 8 minutes. The resulting clear supernatant liquid is poured off and the pellet is discarded. The supernatant liquid in cellulose dialysis tubing is dialysed against a 100xvolume aqueous medium of 0.01 *M* ammonium bicarbonate overnight at 5°C. The dialysed material is lyophilized. The lyophilized extract is dissolved in the minimum amount of PBS for placement on a chromatographic column loaded with Biorad's Biogel A-5M, to achieve a separation of SZP from lower molecular weight species having a molecular weight below 5×10^6. Active fractions, eluted with PBS (fractions comprising cow-SZP) are identified by immunodiffusion and *in vitro* fertilization assay. The active fractions are then placed on a chromatographic column loaded with Biogel A50M, which separates over the molecular weight range 10^6 to 5×10^7. The active fractions are again identified by immunodiffusion and *in vitro* fertilization assay. The active fractions, so identified, give a single band against anti-SZP serum (mouse) which intersects the band found with hamster-SZP, indicating immunological identity. The active fractions block hamster egg fertilization *in vitro*. The resulting fractions comprising cow-SZP in solution are lyophilized for later use.

A vaccine suitable for immunological control of fertility in the human female in unit dosage form by intramuscular injection is prepared by dissolving 500 μg of the above-prepared cow-SZP (lyophilized form) in 1.0 ml. of sterile PBS solution.

WHAT WE CLAIM IS:—

1. Solubilized zona pellucida as hereinbefore defined.

2. A vaccine for immunological control of fertility in the female mammal

comprising solubilized zona pellucida as hereinbefore defined together with a carrier or diluent.

3. An antiserum composition comprising the antibody, anti-solubilized zona pellucida together with a carrier or diluent.

4. An injectable pharmaceutical composition comprising anti-solubilized zona pellucida for control of fertility in the female mammal together with an injectable carrier or diluent.

5. A process for preparing an anti-fertility vaccine comprising solubilizing zona pellucida in an aqueous medium by heating the zona pellucida at from 65 to 100°C. and dissolving the resulting product in water.

6. A method for the immunological control of fertility in a non-human female mammal comprising administering by injection solubilized zona pellucida as hereinbefore defined.

7. A method for the immunological control of fertility in a non-human female mammal comprising administering by injection anti-solubilized zona pellucida.

8. A process as claimed in Claim 5, substantially as hereinbefore described in Examples 1 or 2.

For the Applicants,
D. YOUNG & CO.,
Chartered Patent Agents,
9 & 10 Staple Inn,
London, WC1V 7RD.

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